Nixon. E.L.

BULLETIN No. 212 APRIL, 1927

The Migration of Bacillus amylovorus in Apple Tissue and Its Effect on the Host Cells

(Technical Bulletin)

THE PENNSYLVANIA STATE COLLEGE
SCHOOL of AGRICULTURE and EXPERIMENT STATION
STATE COLLEGE, PENNSYLVANIA

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The Migration of Bacillus amylovorus in Apple Tissue and Its Effects on the Host Cells*

E. L. NIXON

Review of Literature

THE HISTORY of the early speculations as to the cause of Fire Blight has been reviewed frequently and thoroughly (Arthur 2, Stewart 7) and need not be summarized here. Burrill (1) in his epoch-making paper announcing the discovery of the causal organism *Bacillus amylovorus* quoted the American Agriculturist as saying, "The cause of blight is not known. Though the topic is discussed at pomological meetings more than any other, scarcely any positive knowledge has thus far been elicited" and added, "This was literally true and has been repeated in substance hundreds of times until this day. Can it be written hereafter with equal truthfulness?"

Concerning the relations of the organism to the host tissues, Burrill says that the most conspicuous change which can be observed in the tissues affected is the disappearance of stored starch. The cell-walls are not dissolved or in any way altered except in later stages of the disease, when they are stained from oxygenized material. The protoplasm of young cells remains until death occurs. In older cells, the thin protoplasmic lining of the walls can be made out after the starch has all disappeared. "Unless the bacterium does dissolve for itself a passage-way through the cell-wall, it cannot possibly gain entrance in the adult state; yet they are found swarming in cells absolutely closed so far as a power of one thousand diameters can reveal." During the course of its development, according to Burrill, the organism assumes various shapes. "What seems to be the most characteristic form consists of two oblong joints with rounded ends." He suggests that the germs are ultra-microscopical and "that in this condition they pass in water the cellulose barrier developing into visibility within; or that, in the germ condition, they are of such plastic consistency that, amoeba-like, they are able to creep through the narrow spaces between the molecules." This conception was based on Nageli's theory of the micellar structure of the cell-wall. In very young tissues, such as the tips of apple shoots, all parts except the epidermis seem to be affected equally. In older limbs, the chlorophyll-bearing parenchyma of the bark is the first and usually the chief seat of the disease. The bast is not affected, and not infrequently healthy and diseased parts are separated by a concentric layer of bast cells. Sometimes invasion through such a closed layer is accomplished by way of the medullary rays. Contrary to the usual opinion, the cambium is by no means the seat of the disease. This layer of thin-walled cells often retains its vitality when all outside of it perishes. There is no evidence of the progression of the disease in the wood itself. The leaves are invaded from the bark and doubtless may be also the starting point of infection.

According to Arthur (2) the most characteristic feature in the life history of *Micrococcus amylovorus* is the formation of zoögloea. He states, however, that "These have never been observed in the tissues of the tree under any conditions or in

*This work was begun at Columbia University, and has been continued at The Pennsylvania State College. The writer is especially indebted to Professor R. A. Harper for advice and suggestions. Acknowledgment is also made to the officers and botanical staff of The Pennsylvania State College for their assistance and cooperation.

or upon any sort of solid media, but they occur with much regularity in fluid cultures." Arthur discusses the action of the organism in the living plant and states "that a mucilage or gum, which is soluble in water, is produced in abundance, with the disengagement of carbon dioxide. The contents of the cells, together with the cell-walls which have not been liquefied or changed into stony tissue, pass over into this viscous product." He also (Arthur 3) says, "The changes induced in the tissues of the tree partake of the nature of a true fermentation. The bacteria in the tree first attack the starch of the cells, then the cellulose of the cell-walls, and finally the whole tissue becomes a liquid mass. When, however, the cell-walls consist of lignin or other secondary substance, they are not broken down."

Waite (4) states that the inner bark and cambium layers are killed; the bacteria rarely kill the leaves, only occasionally attacking "the stems and midribs." He reports that the disease rarely extends farther than two or three inches from the point of attack in one day, but occasionally travels as much as one foot. He also says, "in certain cases the blight keeps up a sort of slow battle with the tree throughout the summer, so that at the close of the season, when the tree goes into a dormant condition, active blight is still at work in it. The germs keep alive along the advancing margin of the blighted area and although their development is slow it is continuous." Finally in old dried bark, where the disease has stopped, the bacteria have all died and disappeared.

Whetzel and Stewart (5) say that the bacteria slowly work their way through and between the cells of the bark, spreading thus into healthy parts of the twig or limb, killing it. They are able, even under the most favorable conditions, to penetrate but a few inches a day. The bacteria remain alive only along the margin of the canker next to the healthy bark and only in those cankers that are more or less protected from drying out. With spring conditions, the hold-over cankers become active, the bacteria multiply and spread into the adjoining healthy bark, ultimately oozing to the surface.

D. H. Jones (6) states that the bacillus lives in the cells of the inner bark and, as it develops and multiplies, passes along from cell to cell, destroying the tissue as it progresses. It may travel down the twig at the rate of one to two inches per day. The germs present consume all the food material in the affected area and unable to get more, owing to the resistance of the tissue to their invasion, they gradually die from lack of nourishment Sometimes germs will have pushed beyond the well-defined cankered area and will remain alive but not very active in the apparently healthy tissue until favorable conditions once more obtain, when they will resume their activity and thereby produce another cankered area surrounding the old one.

Stewart (7) says after their introduction into the tissues, the organisms multiply rapidly and extend in all directions from the point of entrance. The cells break and split apart, forming pockets that are filled with bacteria suspended in a sticky substance, "presumably a mixture of cell sap and the decomposed substances of the tissues." On the entrance of the organism into the cells Stewart says, "with no apparent openings in the cell-wall that would permit the passage of the bacteria, it may be assumed that the bacteria, push or dissolve their way through thin places in the wall or through pits by mass action."

Peirce (8) states that in root-tubercles the "bacteria are able to soften or dissolve the cell-wall and, when they come into contact with a root-hair, enter it

whether it is broken or not." Having entered the root-hair by softening or dissolving a small portion of the cell-wall and moving or growing through this, the tubercle bacteria multiply rapidly, forming a thread-like zoögloea extending into the epidermal cell. From the epidermis the infecting zoögloea grows fairly straight into the underlying cortical parenchyma, toward the conducting tissues. The direction of the infection thread, he thinks, is determined by attraction exerted by the host-cells upon the bacteria. The infection strands grow definitely, chemotropically, through the cells and also toward the nuclei of the cells which they penetrate.

Smith (9), discussing Peirce's paper, states that "the most striking example, perhaps, is the voluminous intracellular occupation in the root-nodules of Leguminosae. Here the cells are often crowded with bacteria with no visible opening for entrance. They seem to enter by mass action, the bacteria being compacted into strands. Often there is a trumpet-like expansion where the strand touches the cell-wall. The writer has seen what appears to him to be a similar dense occupation of unruptured cells in the bark of the pear attacked by *Bacillus amylovorus*. In a few cases it has seemed as if the bacteria could actually be traced from one cell into another across a narrow pit, but of the absolute correctness of this view I have not yet fully satisfied myself. Probably by mass-action the bacteria push or dissolve their way through pits or similar very thin places in the wall, but the demonstration is hedged about with difficulties. Some of them are at the limit of vision, and might perhaps enter through openings too small to be seen."

Miss Bachman's (10) conclusions concerning the relation of the organism to the host tissues are as follows:

"The bacteria are surrounded by a film of liquid which it seems likely has been drawn out from the cell sap." This film of liquid in which the bacteria move is not extracted in such amount that it precedes the bacteria to any extent. The cells die apparently because of a loss of water although chemical changes in the protoplast may accompany this loss. She concludes that the path of migration is through the intercellular spaces, though she points out that the bacteria enter the xylem tubes very readily. It is evident, she says, that the large intercellular spaces of the cortex provide a ready path for the migration of the organism, though they migrate just as readily between the cells of the pith. She thinks, however, that the organism does not gain entrance to this region readily unless the wound made by inoculation has extended through the bundle ring. Miss Bachman reports no data on the rate at which the bacteria migrate, except to say that forty-eight hours after inoculating water shoots, the bacteria were found one-half inch from the point of inoculation. She does not observe a diminution in the amount of starch in the cells of apple. The cellulose walls are not digested early and if there is a process of cellulose digestion it certainly goes on very slowly and not uniformly over all surfaces. Since the bacteria are most abundant in the intercellular spaces she points out that, "It is possible that we may have here the result of what Smith terms a mass action of bacteria, i. e., a result which can only be brought about by a large number of organisms." She thinks it is possible to explain the broken walls on a purely physical basis. It may be also "that the osmotic pressure of the substance in which the bacteria are found is sufficiently great to rupture the cell-walls" and indeed, in Miss Bachman's judgment, this is the most likely explanation of their final rupture.

Concerning the migration of bacteria in the blossoms of pears, Waite (11)

finds that the bacteria grow in the nectar and multiply there as saprophytes and then "enter the tissues." Smith (9) finds in olive tubercle, bacterial cavities occurring and the organism abundant in them, and easily observed "wedging its way between the cells." In Black Spot on green Japanese plum, he says the earliest stage is simply a "bacterial occupation" of the sub-stomatic chamber. A little later a few neighboring cells "are involved"; with gradual multiplication of the bacteria deeper tissues are involved. A small closed cavity filled with bacteria is formed. On the fruit the spot bulges slightly from pressure of the growing bacterial mass underneath, and gradually "the bacteria burrow deeper and especially wider."

Speaking generally he says, "the wounded surface . . . affords a very suitable soil for the right sort of bacteria. Here it first makes a little growth at the expense of the extruded cell-contents, i. e., it multiplies first of all in the dead tissues of the wound . . . then through the intercellular spaces and the neighboring vessels there is an open passage way into the depth of the tissues, a way which the parasite is not slow to make use of. Enzymes, toxins, acids, and various by-products of the bacterial growth also undoubtedly play their part, weakening the cells of the host or destroying them outright . . . The bacteria multiply, more and more, obstructing some tissues and dissolving, displacing, and crushing others. Cells are separated, cell-walls are softened or dissolved, protoplasm, amids, acids, starch and sugar consumed. Beginning, therefore, with a tiny superficial nidus a parasite gradually burrows its way into the deeper tissue."

Notwithstanding the early discovery of the organism causing blight of the pome fruits as the first known bacterial disease of plants and the considerable amount of very excellent work which has been done on the cause of the disease and the behavior of the bacteria in the tissues, many problems still remain unsolved. It is still not clear just how the bacteria migrate in the intercellular spaces. Much less is known as to how they gain entrance to the cells. The initial behavior of the bacilli, with relation to the crushed and adjacent uninjured cells when they are introduced into a wound, has never been described. Do they form zoögloea or are they merely floating in the extruded plant juices? The ultimate fate of the bacteria in the dead host tissue and on the margin of active cankers is still uncertain. There can be no question that fire blight is especially favorable for the study of host-parasite relations and it was with the hope of contributing to the solution of these and similar problems that I took up its study.

In 1915 at the suggestion of Dr. R. A. Harper of Columbia University, I undertook to determine the history of the organisms from the time of their introduction into the plant by needle point infection until canker formation, and their effects on the host cells of the various tissues through which they pass.

Material and Methods

I have studied chiefly the water sprouts of Early Transparent, Stayman, Williams, and Ben Davis varieties of apple. Two hundred and sixty-seven inoculations were made, and small blocks of young tissue, taken in the vicinity of the puncture, and to a considerable extent down the sprout, were imbedded. The inoculations were made in order to obtain controlled material for the study of the first stages in the invasion of the tissues by the organism. The material was fixed and imbedded at various intervals after inoculation, from a few seconds to nine days. The series included specimens fixed immediately after inoculation, after thirty minutes, after one, three, five, eight, twelve and twenty-four hours,

and thereafter at intervals of twenty-four hours. For studying the relation of the organism to the host tissue it was found highly important to have the very early stages. For comparison, checks of similar tissues punctured with sterile needles, and also of unpunctured normal tissues were imbedded.

The blight cankers for this study were obtained from the base of young water sprouts where they developed naturally following inoculation at the tips. The varieties used were Transcendent Crab, Stayman, Transparent, and Rome. Imbeddings of such cankers were made at intervals of twenty-four hours up to ten days after inoculating. Tissue from dormant cankers was imbedded immediately after being cut from the tree and also after intervals of three, six, eight, and twelve hours in the moist chamber at room temperature. In these studies material was examined from forty-seven cankers.

The fixing of the succulent material was done mostly with Fleming's strong solution, and to a less extent with Fleming's weak solution; most of the tissue from the cankers was fixed with Benda's solution. For staining, Fleming's triple stain was found to be extremely well adapted to differentiate the bacteria. I have found relatively long exposures best for safranin, from fifteen minutes to overnight; comparatively short treatment, one to four minutes depending on the length of the safranin treatment, with gentian violet; and about one minute's treatment with aqueous orange G., or slightly shorter with alcoholic orange G. In general, the exposures to stains were modified according to the age of the tissue, the treatment being shortened for the older tissue. Precautions were taken against overstaining, since destaining proved unsatisfactory for bringing out the bacteria clearly. A thickness of seven microns was found in general to be most favorable for sectioning.

Observations

As is quite universally known, fire blight occurs in the following forms and stages:

1. Blossom blight, which may involve the entire blossom cluster, fruit spur, and, more rarely, girdle or form cankers on the adjacent larger limbs.
2. Twig blight, including blight of water sprouts, which is more often responsible for the formation of hold-over cankers. Most of the water sprout and twig infections die out, however, without forming hold-over cankers.
3. Canker blight, which is a hold-over form, usually occurring at the base of water sprouts, fruit spurs, or small limbs through which the blight organism entered to form the canker.
4. Collar blight, which, in reality, is a hold-over canker form.
5. Root blight, which most often is a hold-over form. In the last two forms the blight organism has many modes of entrance.

I am presenting in this paper my studies on the second and third forms as here listed, beginning with the earliest needle point infection of the water sprout and tracing the invasion through to canker formation. Consideration is given to those instances in which the invasion stops before reaching older tissue and the infection dies out as well as those cases in which older tissue is invaded and hold-over cankers formed.

The first phase of the work undertaken was to determine how the organism, introduced on the needle, travels from the lacerated cells along the path of the needle into the uninjured tissue. A large number of needle inoculations was made,

and at 48-hour intervals blocks of tissue containing the punctures were imbedded and sectioned. In every case it was found that the bacteria had advanced, not only from the puncture into the healthy tissue, but also that in the intervals they had migrated beyond the boundaries of the block. It was found at once that an ordinary laboratory needle makes a needlessly gross and lacerated wound and that if early invasions were to be studied finer needles would have to be used to introduce the inoculum, and that the material would have to be fixed much sooner after inoculation than 48 hours. A large number of inoculations were made then, using a fine insect needle and, as noted, material was fixed and imbedded immediately after inoculation and at intervals of one-half hour, one hour, 3, 5, 8, 12, 24 hours, etc. No attempt was made to control the number of organisms introduced, i. e., the "dose of the infecting agent." That the dosage was fairly small was evidenced by the small number of bacteria later seen in the path of the needle. As few as 5 to 7 per clump were observed. In making the inoculation the needle was inserted to various depths to determine the possiblity of invasion by the parasite in all the tissues of the twig from the cortex on one side through the vascular ring, and pith and the cortex on the other side.

The Formation of Zoögloea.—The major difficulty to be avoided in staining is over-staining the wounded tissue and thus obscuring the bacteria. In properly controlled preparations it is possible to see the bacteria distributed along the path of the needle in all the tissue present at this young stage of the growth of the twig. For the first 30 or 45 minutes after inoculation the bacteria apparently lie just as they were distributed by the needle (Fig. 1). There is certainly very little, if any, movement in the nature of a massing together on the part of the bacteria. Quite commonly small clumps of bacteria can be seen in various tissues throughout the puncture, but they are of no greater frequency than one might expect from the nature of the inoculum. Approximately one hour after the inoculation some of these clumps of bacteria appear to be imbedded in a jelly-like substance somewhat irregular in shape but with a well defined outline (Fig. 2). This jelly-like mass can be referred to properly as zoögloea.

By a study of the consistency and staining qualities of the advancing tips of the zoögloea, or of the whole mass in its very early stages, it becomes evident that its substance is more than a "watery liquid" drawn out of the cells (Figs. 3-4-5-6).

The bacteria are plainly to be seen imbedded in what appears to be a mass of jelly. With the triple stain a beautiful differentiation is given. The jelly always stains a pale blue and shows a finely granular or almost homogenous structure, while the bacteria stain a bright red. In the advancing tips or at the beginning of invasion, the jelly is filled only sparsely by bacteria. A hyaline zone is seen to surround each individual bacillus as it lies in the jelly of the young zoogloea, and, when several are close together, a single hyaline area may enclose them all, thus surrounding from 1 to 4 or 5 bacterial cells in a single envelope. This hyaline envelope is of considerable thickness. Its inner surface follows closely the contour of the contained bacterial cell; the outer surface of the envelope is little more than the surface of the surrounding jelly. As one follows the pseudopod-like arms or branches of the zoögloea back toward the point of inoculation the bacteria become more and more numerous until finally there is not much else visible in the mass.

Migration in the Form of Zoögloea.—This zoögloea mass always forms on the margin of the puncture among the lacerated cells, frequently extending into the

opening caused by the needle but only occasionally filling the cavity entirely. Without exception it is first developed in the cortical region, about ten cells from the epidermis. From this point it advances through the twig in all directions, radially, tangentially, and longitudinally. This movement involves the formation of pseudopod-like projections into the intercellular spaces (Figs. 3-4-5) of the healthy tissue until eventually a complete cylinder of infection involving this portion of the cortex is formed around the twig. The tissues of this region have comparatively large intercellular spaces. The migration radially involves the intercellular spaces of a few cells only; tangentially it involves the complete circumference of the stem; longitudinally it may involve the entire water sprout and adjacent limb, and under optimum conditions may extend several feet. This region of active invasion may be referred to as the region of optimum development or path of migration. From this path the bacteria may extend radially on the one hand until the surface is reached, and on the other until the pith is invaded. This invasion, however, may well be characterized as secondary. Most of the evidence seems to indicate that true intercellular migration occurs only in the "optimum region" and that invasions of the less favorable tissues should be regarded as secondary effects.

As was pointed out, the wound cavities caused by the needle in introducing the inoculum rarely are filled with the bacterial mass. Sometimes, however, apparently under especially favorable conditions, the bacteria multiply in the early zoögloea mass until the entire wound cavity is gorged. It is under such conditions that regions other than the optimum are invaded, e. g., the intercellular spaces of the pith, the vascular bundles, the phloem, etc. This secondary invasion is very much slower than that of the "optimum region." For example, it was found that by the time the pith was invaded a distance of one-eighth of an inch longitudinally from the puncture, the bacteria had formed a complete cylinder around the young twig and had advanced downward three or four inches in the "optimum region" of the cortex. This amount of movement under favorable conditions takes place within 24 to 48 hours after inoculation.

It has been pointed out that the "optimum path" of migration is confined to the intercellular spaces of a zone of cells in the inner cortex, and that after the invasion of this tissue by ramification of the branches of the zoögloca these branches rapidly fill with bacteria. Multiplication continues until the spaces are gorged with bacteria (Figs. 7-8-9). Up to this time there is ordinarily but little migration radially in the stem.

In time, the bacteria become so numerous that they fill all the intercellular spaces of the "optimum region." From the "optimum region" the invasion of adjacent, less favorable tissues, may follow. Sometimes, movement radially outward may develop until masses of the organism break through the epidermis and ooze to the surface in droplets, as the characteristic summer exudate. Toward the interior the organism ultimately traverses radially most of the intercellular spaces of all the tissues of the young stem. The last tissues to be invaded are the cambium, the xylem and pith, and these are rarely seriously attacked. This is a different sort of migration from that which goes on in the "optimum region." It always seems to be associated with the presence of large masses of bacteria in normal intercellular spaces or in schizogenous cavities, suggesting that pressure or "mass action" may play a considerable part in this type of migration (Figs. 8 and 9). I am convinced that these two distinct stages in the invasion of the host must be distinguished. The one involving the penetration of the "optimum region" is the

more rapid and the invasion may travel longitudinally several feet under optimum conditions but it is limited to a single tissue region; the other involving the penetration of the less favorable tissue, is slower, more intense, covering short distances, an inch or so longitudinally at best, but is not limited to one tissue.

The Formation of Schizogenous Cavities.—Immediately following the early invasion of the organism there is no appreciable effect on the host cells. The pseudopod-like tips of the zoögloea can come up to, and pass by, a host cell and have no effect upon it that is visible in any way. In fact, cells are frequently found completely surrounded by bacteria without showing any visible changes due to their presence. Depending somewhat on the condition of the tissue, the first visible effect on the host cells comes within 48 hours after inoculation. This effect might be considered as being the result of a loss of water. There is first a slight, probably toxic plasmolysis, followed finally by the complete collapse of the protoplast but never its dissolution (Figs. 8-9-10). The cell-walls finally yield to pressure, undoubtedly exerted upon them by the bacterial mass, and larger or smaller schizogenous cavities appear, formed by the splitting and separation of the cell walls. Smaller such cavities may become connected with others, making larger and larger cavities.

There is never any indication of the dissolution of the cell-walls or cell contents. Neither is there any evidence that the bacteria enter these cells. Some of the cell-walls, to be sure, are seen to be broken and distorted, but no more than one would expect from the method of formation of the cavities by toxic plasmolysis and crushing. The first destructive effect on the host tissue is that in the "optimum region" of migration. Schizogenous cavities occur here frequently at this stage and often in no other region. I find that without exception the bacteria in these schizogenous cavities, and in the immediately adjacent spaces, finally disintegrate completely, leaving no trace of their presence in the dying tissue of the young sprout. It sometimes happens that the bacteria in other regions invaded by the zoögloea also disintegrate. This, of course, ends further invasion.

Further Migration and the Formation of Lysigenous Cavities.—As the advancing tips of the pseudopod-like branches of the zoögloea are traced downward through the intercellular spaces of the cortical region until older tissue is encountered (especially tissue in which cankers may develop), the bacteria are found to undergo a transformation in size and apparently in functional powers. They change from the characteristic bacilli and become shortened by one-half or more of their usual length, stain deeply, and tend somewhat to be oriented in the same direction. In this form they can be found in the intercellular spaces of all regions of the basal part of the water sprout and adjacent limb, except the hard bast, xylem, pith, and perhaps the cambium. Their intricate ramifications are most conspicuous in the region of the medullary rays. In this tissue, cut in radial longitudinal sections, they give one the impression of forming threads or chains within tubes extending the full length of the ray. The bacteria are invariably oriented lengthwise of the intercellular space which is so small at times that the bacteria are arranged single file.

At various intervals throughout these regions the bacteria are found inside the host cells, a condition which is not found in the typical zoögloea stage (Fig. 11). They ultimately destroy the protoplast with all its contents, together with the cell-walls. Frequently they invade groups of adjacent cells and, breaking them down entirely, form lysigenous cavities of various sizes. This process apparently

involves the complete dissolution of the tissues concerned, leaving irregular and ragged openings (Figs. 12 and 13). In the formation of these cavities there is no indication that crushing pressure is exerted by the bacterial masses. In fact, just the opposite condition seems to prevail. More space is made available than the bacteria occupy. There is always an open marginal zone between the bacterial mass and the surrounding host tissue. These cavities may be found in any of the tissues mentioned above, but are most prevalent in the cortical region, phloem parenchyma (Fig. 13), and medullary rays (Fig. 12). The lysigenous and schizogenous cavities never occur together, though both may develop in the same types of tissue. Neither do the characteristic bacilli of the zoögloea stage nor these reduced forms found in the lysigenous cavities and adjacent intercellular spaces occur together. Just where the line of demarcation between the two stages occurs is still somewhat obscure. It is evident from the preparations studied that this line is drawn fairly sharply, for one block of tissue has shown one condition and the next adjacent block the other. Whether the transformation is due to a cyclic change in the bacteria, or is a direct reaction to the condition of the host, is not entirely clear. The zoögloea stage and the formation of schizogenous cavities are found most commonly in the young succulent parts of the water sprouts, while the reduced stage, with the accompanying lysigenous cavities, occurs with greater regularity in the tissues of the older limbs and trunks. The reverse condition, however, has been observed occasionally.

In studying the disintegrating bacteria in the different infected regions, several distinct forms of bacterial cells were observed. Two or three are typical involution forms, such as are commonly reported. In the other, the bacterial cell contains what is apparently a true spore. I have not yet determined whether the involution forms or the spores may be concerned in continuing the life cycle of the organism. No attempt has been made to determine whether either will reproduce the disease.

Cyst Formation.—Coincident with the formation of lysigenous cavities, the bacteria are to be seen plainly in the lumina of certain cells, particularly those of the phloem parenchyma, cortex and, to a less extent, those of the phloem ray cells. The bacterial mass in these cells has been traced through several stages. At first the bacteria are not numerous, but they stain very densely and are the typical reduced forms, and, as in the earlier stage, are surrounded by a hyaline zone. More conspicuous than the bacteria is the gelatinous substance in which they are imbedded. This gelatinous substance is unusual in abundance and density. Careful comparative study suggests the conclusion that the bacteria in these cells increase rapidly in number. The bacteria then aggregate themselves into a core-like center, often arranged in spiral series (Fig. 14 and 15). This development apparently continues until the entire mass is rounded up and the individual bacteria are no longer distinguishable (Fig. 16).

Since these preparations were made from material which supposedly would form fall over cankers, it seemed logical to assume that such stages were associated with fall over cankers. I next attempted to study these intercellular and intracellular stages in dormant cankers.

Living cankers, formed at the base of the water sprouts in 3 to 6-year-old limbs, accordingly were procured in zero weather. Of these, some blocks were imbedded immediately and others at intervals after having been placed in the moist chamber as noted above. When the dormant material was sectioned, it was found that no free individual bacilli, either in intercellular spaces or in cavities, could be

found. The line between the disintegrating tissue and apparently healthy tissue was drawn quite sharply. In the disorganized tissue (lysigenous cavities) all the bacteria, once so abundant, appeared to have completely disappeared.

In the apparently living tissues, large oval, dark-staining, more or less granular masses in the lumina of certain cells were, however, conspicuous (Fig. 17). These as shown by subsequent study, are the encysted masses of the bacteria and apparently constitute the normal winter condition in cankers which will become active the following spring. Adjacent blocks taken successively after the cankers had been placed in the moist chamber for various periods, when sectioned, revealed the presence of masses of bacteria in the place of the round, dark-staining intracellular cysts (Fig. 18). These masses have been teased out of fresh tissue repeatedly and have been observed to break up in a drop of distilled water under the microscope. When this material was used as an inoculum in young apple shoots, typical blighting resulted. I am applying the term cyst to these bacterial masses. The bacterial cells in the cysts are packed so closely together that their individual identification is quite impossible, even when examined in sections three microns in thickness. Upon the application of water, the encysted masses disintegrate so rapidly that one has to be quick to see them actually break up. From these encysted masses emerges the true bacillus form of the organism, not unlike in any way those of the zoögloea stage.

The behavior of these cysts in the spring development of a hold-over canker is made quite clear from a study of the preparations made from material placed in the moist chamber for different intervals. In the spring, the encysted masses in the natural cankers imbibe moisture and liberate their bacteria. In doing so, the masses swell to several times their former size, break out of the cells, join those from other cells, and migrate outward to the surface as the well-known canker exudate. These cysts are distinguishable plainly from other cell inclusions in the various regions of the host tissue, by their appearance as well as by their behavior when moistened.

Cyst germination completes the normal cycle of development from infection to infection, and under natural conditions covers a period of one year. Not all infections, however, complete this developmental cycle. As previously pointed out, all of the bacteria arising from a given infection under some conditions may disintegrate. In some cases invasion terminates with the formation of schizogenous cavities. In others the formation of lysigenous cavities seems to terminate invasion. Transcendent crab furnished the greatest per cent of cankers having the encysted bacteria.

Discussion

Burrill discovered the causal organism, and he unquestionably saw schizogenous cavities in the tissues, but I find no evidence in his report that he saw lysigenous cavities. He was the first to mention the migration of the organism through the medullary rays and he also reported the presence of the bacteria within the cells.

Arthur was the first to report the formation of zoögloea of *B. amylovorus* in liquid media, and the formation of lysigenous cavities.

Miss Bachman's description of the "film of liquid," surrounding the bacteria in the early stages corresponds to that of the zoögloea migrating through the intercellular spaces, as I have found it.

It appears that the actual migration of the organism has been mostly overlooked. This, I think, is due to the fact that the material studied was taken at advanced stages of the disease. When such material is sectioned it shows the bacteria well distributed throughout the tissues but does not reveal the manner of migration. The failure of recent investigations to give a connected story of the behavior of this organism is due largely to the practice of "killing" and fixing material already dead, or to conclusions based too largely on purely macroscopic evidence.

I have used the term zoögloea for the migrating mass of bacteria. The term zoögloea as used by bacteriologists refers to a colony formed of individual bacteria imbedded in what appears to be a mass of jelly. The bacteria are held together by a matrix secreted by themselves. Peirce has used the term for the migrating strands and masses of *B. radicicola* in the nodules of the Leguminosae. Robinson and Walkden (12) have used it for the intercellular migrating stage of *B. tumefaciens* in the formation of crown gall in plants. Arthur uses it for the irregular, though definitely outlined, masses of bacteria which occurred in his cultures of *B. amylovorus* in fluid media.

Many attempts have been made to explain the migration of the organism through the host tissue. In the literature on this subject, such statements as the following are found: "They work their way through and between the cells." "They extend in all directions." "They pass along from cell to cell." "The large intercellular spaces in the cortex provide a ready path for their migration." They enter the tissue." "They wedge their way between the cells." "Pressure is exerted." "A bacterial occupation is brought about." "They burrow their way."

In my study I have followed the advance of the organism in its earliest stages, from the point of its introduction in the needle puncture, and I find that the migrating organisms are always imbedded in dense jelly, forming a zoögloea. The movement is doubtless analagous to that by which the myxobacteriaceae build their complex sorocarps, and parallels in many respects the movements of pseudoplasmodia.

As noted, the bacteria can be seen clearly, distributed all along the path of the needle, but the actual tissue infected is quite localized. This fact has, undoubtedly, both an anatomical and physiological basis. The significant thing is that the initial position of the zoögloea mass is not determined by the mechanical distribution of the inoculum, but depends upon a special relationship between the organism and the host tissue. This relationship may be thought of as involving both a selective power on the part of the invading organism and a special susceptibility of the cortical tissues, due in part possibly to their relatively undifferentiated condition and their high food content. These same interrelations doubtless determine the extensive migration in what I have called the "optimum region."

That the spread of the bacteria through the host tissue is due merely to mechanical pressure, developed between the host tissue and the growing mass of parasites, is certainly not in accordance with the obvious facts as shown in these sections. On the pressure idea, one would have considerable difficulty in explaining how the zoögloea mass originating on the margin of a comparatively large wound would be able to penetrate the intercellular spaces of the lacerated cells and finally the intercellular spaces of the adjacent healthy tissue without first filling the wound cavity more or less completely. The fact is that the wound cavities rarely are filled with the bacterial mass (Fig. 2). In studying the advancing tips of the

branching zoögloea as they penetrate the large intercellular spaces of the cortical region one finds abundant evidence that mutual pressure is not responsible for their forward movement. Their tips are blunt, well defined, and creep past lateral spaces as though they were under no great pressure. They fail to flow into large cavities that they would certainly occupy before making further progress if pressure were responsible for their movements (Fig. 5).

That the organism has the power to dissolve the cell-wall, as well as the cell contents, is evidenced by the large lysigenous cavities. The bacteria evidently may gain entrance to the cell by dissolving a passage way through the cell-wall. Many openings of greater or less diameter have been observed. Most of these, however, I believe, are too far advanced to determine exactly how the operation is accomplished. Masses of the reduced form of the bacteria can be traced through intercellular spaces, past several cells until suddenly one comes to a cell that has a very small number of bacteria inside the wall. There is no evidence in my preparations to show that this entrance into the cell is accomplished by infection threads, but there is abundant evidence to show that, ultimately at least, openings are formed by solution of the wall. To determine whether the first few bacteria that enter do so by dissolving a region of the wall is not easy. When whole groups of cells with their walls and contents are dissolved in the formation of lysigenous cavities there should be no skepticism concerning the dissolution of the wall. On the entrance of *Polyangium parasiticum*, one of the myxobacteriaceae, into the cells of Cladophora, Geitler (13) reports that the bacterial mass attacks the outside membrane, separates it into layers, and through successive dissolution of the layers effects an opening which is large enough to serve as a place of entry. He says the protoplast of the host at first reacts by slight plasmolysis which later follows rapidly on all sides. If the bacterial mass is very large, it may penetrate several cells of the filament, even dissolving cross-walls. In digesting the host cell, plasma, nucleus, chromatophores, pyrenoids, and starch are attacked equally. Usually the cell membranes are resistant, although at times these are dissolved. For the most part they are dissolved just enough to permit entrance of the bacterial mass. He adds that, in the penetration of the cell, the membrane is separated into layers, of which the inner one is much perforated, like a sieve. It remains unknown whether the fine cross-stripes in the membrane are fine canals resulting from solution by a single bacillus, or whether the bacilli bore through the membane. As the membrane becomes thinner and thinner, and the holes become larger, it finally falls to pieces. In the beginning of this whole process the bacilli become oriented perpendicularly to the cell-walls, which he says is undoubtedly the condition for beginning the penetration. There are several other points of similarity between the behavior of this organism as reported by Geitler, and my finding for *B. amylovorus*. In *P. parasiticum* he reports the vegetative stage as saprophytic; the reproductive as parasitic. In *B. amylovorus* the vegetative is intercellular; the reproductive intracellular. In each there is a reduction in size of the bacteria as the reproductive stage is approached. Without this change of phase no penetration of cells nor cyst development occurs in either. The bacilli multiply by fission, and in both forms they are surrounded by a thin layer of hyaline slime. He finds that the entire process from beginning of penetration to cyst formation requires about 42 hours.

Thaxter (14), in his discussion of the life history of the myxobacteriaceae, notes several points with which my studies of *B. amylovorus* are in striking agreement.

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It should be noted, he says, that the life history of these organisms shows a distinct and more or less regular division into two periods. In the first period of vegetation, under favorable conditions there is a swarm or collection of rod-like bodies, always distinct from one another, having power of slow locomotion and secreting as they multiply (by fission) a firm gelatinous base which connects the colony as a whole. It always possesses a clearly defined advancing edge or border. The colony continues to extend itself in this fashion while conditions remain favorable for growth; but finally individuals within the advancing border, having increased rapidly for a certain period, begin to swarm together at different points. As the bacilli run together to produce cysts, they become thickened and somewhat shorter. This piling up of individuals marks the beginning, he says, of the second period, a fructification or pseudo-fructification which has for its object the production of a resting stage. In the simplest forms a gelatinous envelope becomes hardened about them to form a protecting wall.

Conclusions

1. *B. amylovorus* migrates through the apple tissue in the form of a zoögloea. In the early stages, the most extensive migration is intercellular. Due to the action of the organism, two distinct types of ultimate injury result; the formation of schizogenous and lysigenous cavities.

2. In the life cycle of *B. amylovorus*, there are two phases or periods: a vegetative stage and a pseudo-fructification. The major portion of the intercellular migration in the form of zoögloea comprises the vegetative phase. Intracellular migration with, ultimately, the formation of cysts, characterizes the pseudo-fructification phase. The cysts represent the winter condition of the bacteria. In these respects the life history of *B. amylovorus* is quite similar to that of the lower forms of the myxobacteriaceae.

Description of Illustrations

FIGURES 1-10

The Vegetative Stage of *B. amylovorus* culminating in the formation of schizogenous cavities.

Fig. 1.—A cross-section of a young water sprout showing the bacteria as left in the path of the needle after inoculating.

Fig. 2.—The development of a young zoögloea on the edge of the puncture starting infection in the cortical region about ten cells from the epidermis, one hour after inoculating.

Fig. 3.—Showing the advancing margin of the zoögloea as it moves into the intercellular spaces in the optimum region of migration, 24 hours after inoculating.

Fig. 4.—Showing in cross-section the advancing margin of the zoögloea as it moves into the intercellular spaces, 24 hours after inoculating.

Fig. 5.—Showing in longitudinal section the pseudopod-like branch following an intercellular space, 24 hours after inoculating.

Fig. 6—Tip of Fig. 4 in detail showing texture of zoögloea and hyaline areas around each bacillus.

Fig. 7.—A streaming or flowing of the bacterial mass at time of the oozing to the surface as the characteristic summer exudate, 48 hours after inoculating.

Figs. 8 and 9.—Stages in the formation of schizogenous cavities, showing death to some cells, 72 hours after inoculating.

Fig. 10.—Death of the tissue and no bacteria inside the cells, 72 to 96 hours after inoculating.

FIGURES 11-18

The Reproductive Period of *B. amylovorus* beginning with intercellular invasion and destruction, and culminating with intracellular pseudo-frutification.

Fig. 11.—Invasion of the cell lumen and invagination of the protoplast.

Fig. 12.—The formation of a lysigenous cavity.

Fig. 13.—Showing the destruction of several cells resulting in the formation of a larger cavity.

Figs. 14 and 15.—Early stages in the formation of cysts, with the circular or spiral development of the bacteria into a core-like center.

Fig. 16.—The protoplasmic mass surrounded with bacteria. A late stage in cyst formation.

Fig. 17.—A dormant cyst.

Fig. 18.—The early dissolution of cysts, six hours after placing the canker in the moist chamber.

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